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14. ABSTRACT
The Research Project supported by this DOD PCRP Physician Research Training Award investigates novel biomarkers for prostate cancer detection and prediction of disease outcome. The goals and objectives of this study are summarized by the Specific Aims: 1. Evaluate the relative levels of expression of our panel of candidate protein biomarkers in urine, tissue and serum from patients with prostate cancer compared with normal controls to identify prostate cancer specific biomarkers. 2. Evaluate the relative urine, tissue and serum levels of these prostate cancer specific biomarkers within our entire active surveillance (AS) cohort to identify accurate biomarkers predictive of indolent vs. progressive prostate cancer. The funding from this Physician Research Training Award provides salary support for Dr. Adam S. Feldman to secure protected time as a translational and clinical investigator in prostate cancer research. It also provides salary support for a Research Assistant for this project.

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Introduction:

The Research Project supported by this DOD PCRP Physician Research Training Award investigates novel biomarkers for prostate cancer detection and prediction of disease outcome. The goals and objectives of this study are summarized by the Specific Aims: 1. Evaluate the relative levels of expression of our panel of candidate protein biomarkers in urine, tissue and serum from patients with prostate cancer compared with normal controls to identify prostate cancer specific biomarkers. 2. Evaluate the relative urine, tissue and serum levels of these prostate cancer specific biomarkers within our entire active surveillance (AS) cohort to identify accurate biomarkers predictive of indolent vs. progressive prostate cancer. The funding from this Physician Research Training Award provides salary support for Dr. Adam S. Feldman to secure protected time as a translational and clinical investigator in prostate cancer research. It also provides salary support for a Research Assistant for this project.

Body:

The first year of my DOD PCRP PRTA was very productive from both a translational laboratory and clinical research standpoint. In summation, I used mass spectrometry (MS) to quantitatively compare the entire urinary proteome and identify differentially expressed proteins in the urine from men with prostate cancer as compared with those found in controls. The MS analysis identified >1400 unique proteins, comparative analysis revealed 55 potential prostate cancer specific proteins, and using bioinformatic database analyses, we narrowed this list to 20 biologically relevant proteins. Using semi-quantitative Western blot, we investigated several proteins on the list of 20 relevant proteins including Leukocyte Elastase Inhibitor, Annexin A1, Plastin-2, Vimentin, and Tissue Inhibitor of Matrix Metalloproteinase Type 1 (TIMP-1). We used urine specimens of 56 men, both from PrCA and Controls. These urine specimens were selected from our urine biospecimen bank, prospectively obtained and developed from our urologic oncology clinic at Massachusetts General Hospital. In TIMP-1, we found a significant difference in TIMP-1 expression between men with Gleason 3+3 disease and men with Gleason 7 or greater.

In this second year of my DOD PCRP PRTA, I further explored the compelling data from the TIMP-1 Western blots and returned to my original list of 55 differentially expressed potential prostate cancer specific proteins. Looking at TIMP-1, we used Enzyme-Linked Immunosorbent Assays (ELISA) and Immunohistochemistry (IHC) to corroborate the data we found in Western blot analyses. Using IHC, we were able to show markedly unique staining for Gleason 8 or

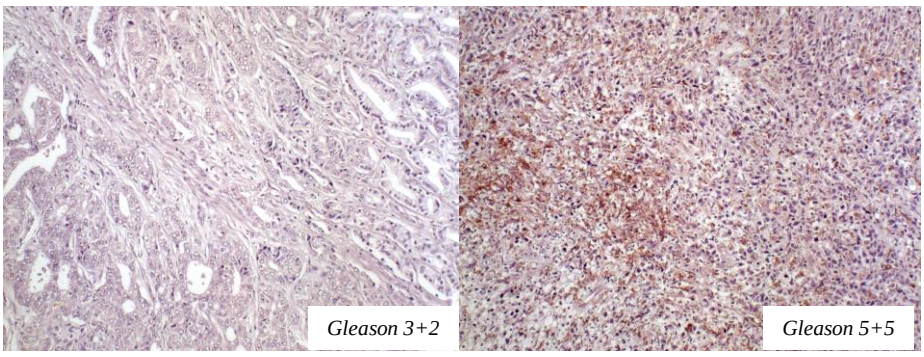

Figure 1: Representative IHC staining for TIMP-1 in prostate cancer tissue.

higher, compared to lower Gleason scores (Figure 1); this supports our previous Western blot data and points to the the potential of TIMP-1 as a relevant biomarker for prostate cancer.

For ELISA analysis of TIMP-1 expression we used the same cohort of 56 men, both PrCA and Controls, as we had analyzed by Western blot. Although we demonstrated differential expression across our cohort, we were unable to effectively reproduce the results we found in Western blot (Figure 2). This discrepancy between Western blot and ELISA results were consistent across two separate commercially available ELISA kits (R&D Systems, Mineapolis, MN and EMD Millipore, Billerica, MA).

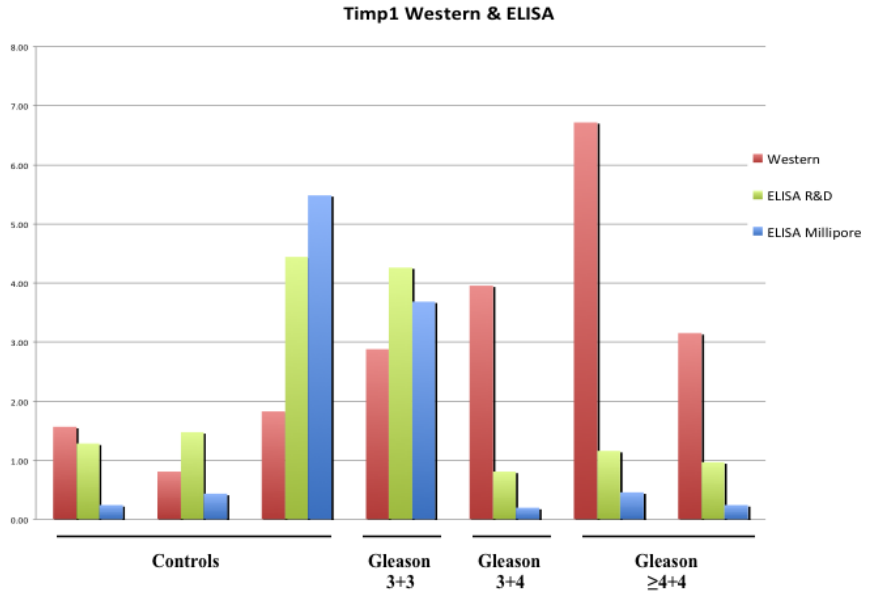

Figure 2: Western blot and ELISA analysis of urinary expression of TIMP-1.

This discrepancy could be caused by a few factors. First, the Western blot procedure requires denaturing of the proteins in the urine samples and ELISA does not. We addressed this issue by using Dithiothreitol (DTT) to denature the proteins in the urine samples for ELISA, but found that the addition of DTT inhibited the efficacy of the ELISA. A second potential issue is the diverse composition of urine itself and the differences in sample preparation required for Western blot compared with that for ELISA. Unlike the Western blot, the ELISA protocol detects protein within a sample of isolated protein. Western blots, however, depend on polyacrylamide gel electrophoresis followed by electromotive transfer of the protein from the gel onto a polyvinylidene difluoride (PVDF) membrane. This process is essentially a built in purification process during Western blot preparation and is not present in ELISA preparation. Therefore, this may affect the detectable presence of low molecular weight and low abundance proteins in a sample and therefore lead to discrepancies in apparent expression as measured by these two methods. We attempted to correct for this issue when processing the urine samples by diluting, and then concentrating to filter out some of the solutes. However, it may be that we cannot successfully purify or prepare the urine samples in an effective manner to replicate the Western blot results.

We also returned to my original list of 55 differentially expressed potential prostate cancer specific proteins, for both Western blot and ELISA analyses. We looked at Prohibitin, Radixin, Taldo1, Fructose-Bisphosphate Aldolase A, Lactate Dehydrogenase A, CD63, Cytochrome C, Ras-related protein RAB-3A, Macrophage Capping Protein, 10kd Heat Shock Protein, Annexin A3, Sorbitol Dehydrogenase, Fibrinogen Beta Chain Precursor, and Creatine Kinase B-Type. As a first pass evaluation of these proteins, we used a single Western blot with several representative specimens as an initial evaluation to screen for those proteins with potential clinical relevance. Blots in this initial screen were evaluated for differences in protein expression by visual inspection of bands. Among the proteins we looked at, Semenogelin-2, Lactoylglutathione Lyase, Hepsin, Leukocyte Elastase Inhibitor (SERPINB1), Alpha-1-Antichymotrypsin (SerpinA3), and Growth-Inhibiting Protein 12 (GIP 12) are the most interesting showing varying protein levels comparing PrCA and Controls, and within the varying Gleason scores of PrCA patients (Figure 3). These proteins show promise and warrant further analyses with a greater sample size of prostate cancer patients and controls. Proteins investigated by Western blot analyses with less impressive data are demonstrated in Figure 4. These include Lactate Dehydrogenase A (LDHA) and Aldolase A, which did not demonstrate any detectable bands in urinary protein, and Anti-Lactoylglutathion Lyase, Plastin-L, and Radixin, which demonstrated some urinary expression, but no definable pattern compared with tumor characteristics. One of our goals is to create a panel with our more promising biomarker candidates.

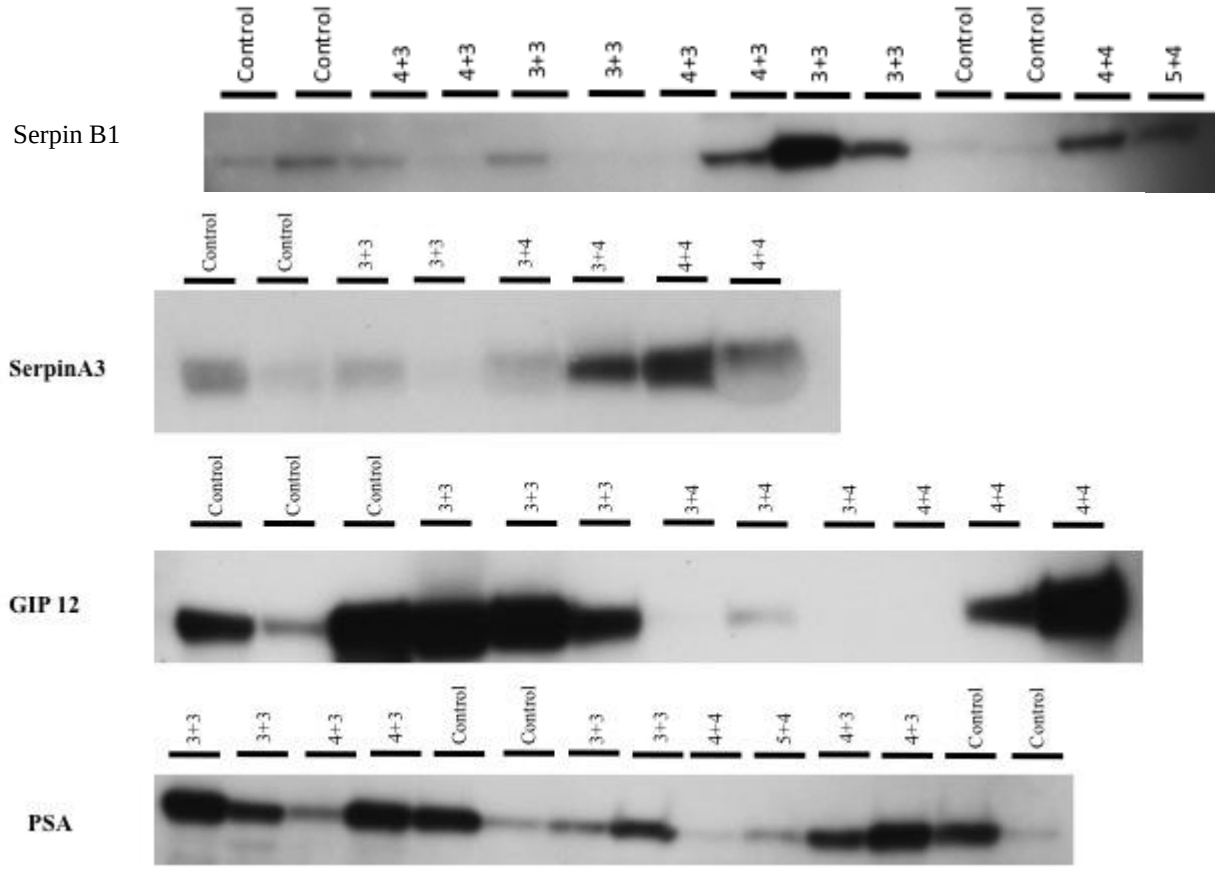

Figure 3: Representative Western blot analysis of urinary expression of Serpin B1, Serpin A3, GIP 12 and PSA.

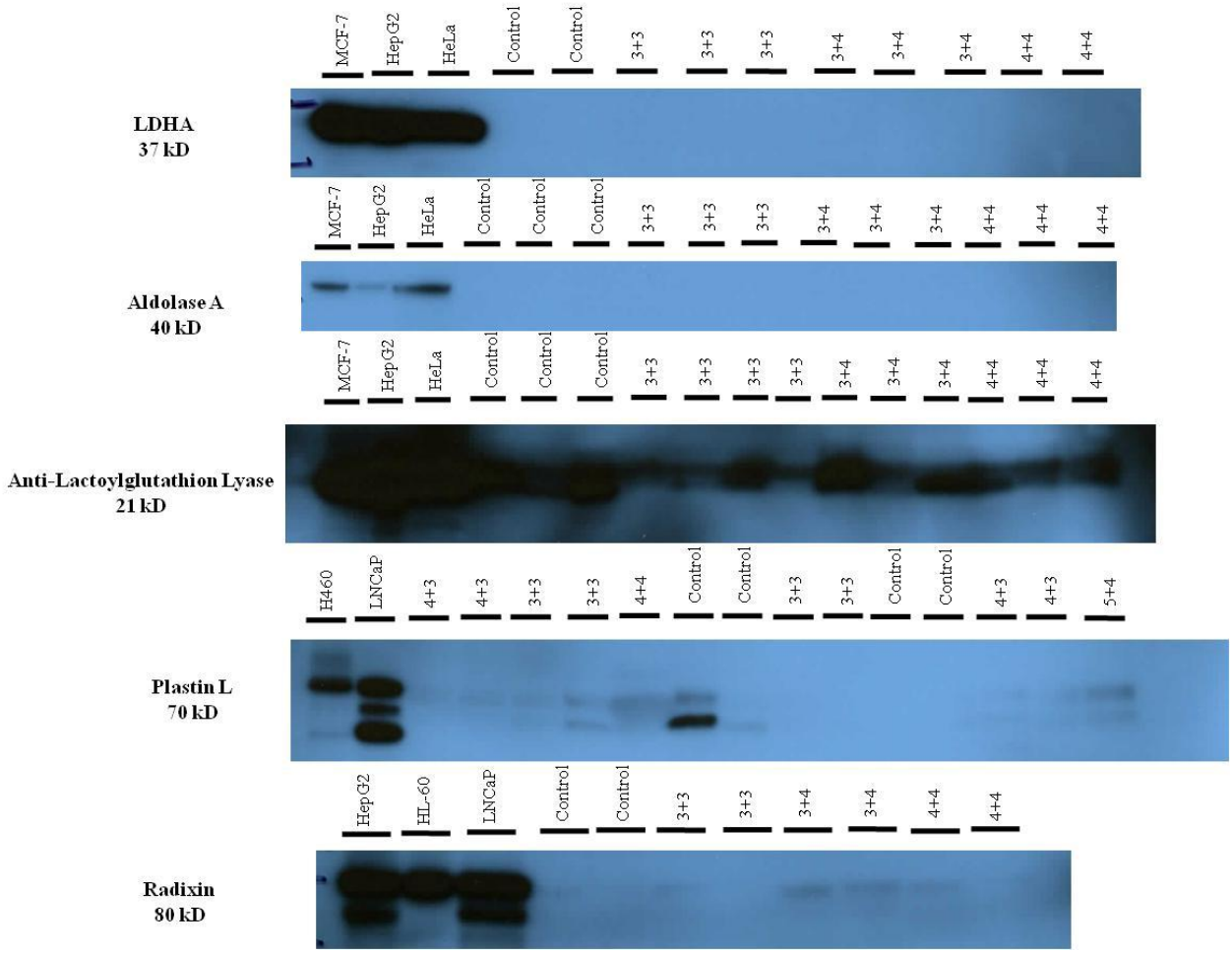

Figure 4: Examples of Western blots from include Lactate Dehydrogenase A (LDHA), Aldolase A, Anti-Lactoylglutathion Lyase, Plastin-L, and Radixin.

In addition to success in the laboratory, we have continued to build on our previous work in evaluating our cohort of men on active surveillance (AS) for low risk prostate cancer. This cohort was identified in year 1 through billing and pathology records and consists of 469 men on AS between 1997 and 2009. Although AS had been practiced throughout this entire period, in 2008 our group agreed upon formal guidelines for AS at our institution. Inclusion criteria included Gleason ≤ 6, Gleason 7 in select patients with low volume, no more than 3/12 cores positive with $\leq$20% in each core, and PSA <10. Our AS follow-up protocol involves PSA testing and a digital rectal examination every four months for one year, followed by every six months for two years, and then annually. Those on AS also have a mandatory repeat 12-core biopsy 12-18 months after initial diagnosis. Additional biopsies are at the discretion of the treating physician.

	Median (range), N (%)	Mean
# Biopsy cores taken	12 (5-22)	10.9
# Positive cores	1 (1-3)	1.38
Post diagnosis biopsy	1 (1-5)	1.5
Number of prostate rebiopsies		
1	308 (65.7%)	
> 1	107 (22.8%)	
> 2	25 (5.3%)	
Pathologic finding on 1st rebiopsy (n=308)		
Atypia	3 (1.0%)	
Benign	67 (21.8%)	
Prostate Cancer	209 (67.9%)	
PIN	29 (9.4%)	
Gleason score progression (from Gleason 6)	55 (17.9%)	
Gleason 3 + 4 = 7	33/55 (60.0%)	
Gleason 4 + 3 = 7	13/55 (23.6%)	
Gleason 8 - 10	9/55 (16.4%)	
Cancer volume progression (from <33% positive cores to ≥33%)	52 (16.8%)	

Table 1: Results of repeat biopsies in Active Surveillance cohort.

On first re-biopsy, prostate cancer was identified in the specimen in 67.9% of the patients (Table 1). Fifty-five (17.9%) experienced an increase in their Gleason score and fifty-two (16.8%) experienced cancer-volume progression, defined as an increase from less than 33% to 33% or more. 116 men (24.7%) progressed to active treatment and within these men, the most common reason for treatment was pathologic progression (44.8), followed by PSA progression (30.2%), patient preference (12.1%), and DRE progression (5.2%). Those men who experienced pathologic progression appeared to have a greater PSA velocity and shorter PSA doubling time, however, we are continuing to investigate this relationship as these results may be confounded by inherent bias.

As reported previously, freedom from intervention at 5 years in our cohort was 77%, and at 10 years was 62%. At both five and 10 years, the cancer-specific survival rate was 100%. The overall survival rate was 95% at five years and 88% at 10 years. We are currently in the process of writing the manuscript to report these data.

In addition to significant research accomplishments, I continue to meet my goals within the training program of this grant. I meet regularly with my two mentors, Drs. Matthew Smith and Bruce Zetter. In our regular meetings, we not only discuss research progress, but also focus on career planning and guidance. I attend regular urologic oncology clinical and research conferences at our institution and both attend and present at regional and national scientific meetings. I attend regular laboratory research meetings both for our own research progress, as well as reviewing other associated research in the current literature. I also have begun participating as an invited reviewer of research grant applications for the Prostate Cancer Foundation.

Key Research Accomplishments:

- Identification of panel of biologically relevant proteins in urine which may be prostate cancer specific.
- Preliminary demonstration that one of our identified proteins, TIMP-1, appears to be more highly expressed in the urine of men with intermediate or high risk disease, in not only Western blot but Immunohistochemistry staining as well.
- Continued analysis of a large database of our cohort of men with low risk prostate cancer on active surveillance.

Reportable Outcomes:

- **Feldman AS**, Fergus M, Smith M, Zetter B. TIMP-1 demonstrates variable expression in urine and tissue of men with and without prostate cancer. Presented at the Prostate Cancer Foundation Scientific Retreat, October 2012.
- Carrasquillo R, Preston M, Coen J, Zietman A, Smith M, Wu CL, McDougal WS, **Feldman AS.** Gleason upgrading and Increased Cancer Volume on Repeat Prostate Biopsy in Patients on Active Surveillance. Abstract presented at the American Urological Association New England Section, October 2012.
- Preston M, Carrasquillo R, Coen J, Zietman A, Smith M, Wu CL, McDougal WS, **Feldman AS.** Need for Intervention and Survival in a Cohort of Patients on Active Surveillance for Low-risk Prostate Cancer. Abstract presented at the American Urological Association New England Section, October 2012.

Conclusion:

In summary, the first two years of my DOD PCRP PRTA have been very productive. We furthered our study of novel TIMP-1 protein in Immunohistochemistry and ELISA analyses. We are also continuing to investigate our list of biologically relevant candidate prostate cancer biomarkers and have once again demonstrated promising results with other candidates. We are moving toward a panel of our biomarkers to rival the current biomarker PSA.

In addition to success in our laboratory work, we have also made significant accomplishments in continued analysis of our large cohort of men on active surveillance for prostate cancer. We are currently in the process of writing the manuscript to publish our results.

This work is very relevant to current clinical practice in prostate cancer and meets any potential "so what" criteria. New diagnostic and predictive biomarkers with improved performance characteristics than prostate specific antigen (PSA) are sorely needed. The work funded by this grant directly addresses that challenge and we are already beginning to produce results toward that goal. In addition, it is clear that we have historically over-treated low risk prostate cancer. Active surveillance is a management strategy for low risk disease which will help ameliorate the problem of overtreatment. Our large database of men on active surveillance will help us to understand the safety, efficacy and outcomes of this strategy and will help us better select men for AS in the future. Biomarker analysis

within this cohort will also help us better understand who truly has very low risk disease and can safely avoid radical treatment.